DRAFT V1.2

VICH DISCUSSION DOCUMENT

Prepared by Hans A. Draayer
Central Research Division
Pfizer Inc
Groton, CT 06340

VICH Biological Quality Monitoring WG
Topic: Residual Formaldehyde Testing

Purpose:

In accordance with the VICH Standard Operating Procedure for Topic Startup, this Discussion Document (DD) outlines regulatory and technical requirements for the testing of residual formaldehyde levels in final biological products. Proposals for harmonization are included as points for discussion. A meeting plan and target dates for submission of draft recommendations to the VICH Steering Committee are included in this DD.

Current Regulations:

A number of assays are available for the determination of residual free formaldehyde in inactivated vaccines. These assays are referenced in the following table:

Procedure/Spec.	EU/CVMP	9 CFR/SAM	JP
References		9 CFR 113.100 (f)	Minimum requirements of Biological Products for Animal Use
	CVMP guideline for inactivated vaccines 1.9.2.4	SAM 510.02	General Test (Formaldehyde determination method)
	EP V2.4.18	USP 541	P 403
Methods	Test for free formaldehyde	Demonstration of lack of viricidal activity or Basic fuchsin (Schiff)	Test for free formaldehyde (Acetyle Acetone method)
Limits	<0.05% unless higher concentration has been shown safe	Clostridials <0.5% Other vaccines <0.2%	Level to be within set limits for the product (<0.01 - <0.5%)
Sample size	not specified	not specified	not specified

Limits for free formaldehyde concentrations range from <0.05% in the EU, <0.01 to <0.5% in Japan and <0.2% to <0.5% in the US depending on the type of product. Greater levels are allowed in the EU if supported by host animal safety data.

Proposed Guideline:

1) If formaldehyde is used as the inactivating agent, (and the product has not been found free of viricidal activity?) bulk or final container samples shall be tested for residual free formaldehyde using one of the following assays:

    - X
    - Y
    - Z

2) *Alternative; If formaldehyde is used as the inactivating agent, (and the product has not been found free of viricidal activity?) bulk or final container samples shall be tested for residual free formaldehyde using x assay. If formulation of the product does not allow use of this assay, one the following may be specified in the manufacturing specifications:*
    - *X*
    - *Y*
    - *Y*

3) Formaldehyde concentration will be presented as w/v% *(alternatively v/v%)*
4) Assay will be validated and described in the manufacturing specifications (Production outline).
5) Products will not exceed <0.05% (?) residual free formaldehyde, unless higher levels have been demonstrated as safe.
6) Maximum allowable levels, with supporting safety data are 0.2% for conventional inactivated biological products and 0.5% for biological products containing Clostridial antigens.
7) *Alternative: Product shall not exceed .5% formaldehyde or the maximum level authorized in the manufacturing specifications and supported by host animal safety data.*

Work Plan

1. Complete Draft DD	complete
2. Submit DD to VICH Biological Quality WG and VICH secretariat	complete
3. Hold first Biologics Quality WG meeting.	2/99
4. Complete draft guideline for review by membership	4/99

Attachments

1. US Code of Federal Regulations (CFR) 9 113.100
2. USDA Standard Assay Method (SAM) 510
3. US Pharmacopoeia 541
4. EU Guideline 111/3181/91 Section 1.9.2.4
5. European Pharmacopoeia 2.4.18.
6. Japanese Standard Requirement

113.100 General requirements for inactivated bacterial products.

Unless otherwise prescribed in an applicable Standard Requirement or in the filed Outline of Production, an inactivated bacterial product shall meet the applicable requirements in this section.

(a) Purity tests. (1) Final container samples of completed product from each serial and each subserial shall be tested for viable bacteria and fungi as provided in 113.26.

(2) Each lot of Master Seed Bacteria shall be tested for the presence of extraneous viable bacteria and fungi in accordance with the test provided in •113.27(d).

(b) Safety tests. Bulk or final container samples of completed product from each serial shall be tested for safety in young adult mice in accordance with the test provided in 113.33(b)
unless:

(1) The product contains material which is inherently lethal for mice. In such instances, the guinea pig safety test provided in •113.38 shall be conducted in place of the mouse safety test.

(2) The product is recommended for poultry. In such instances, the product shall be safety tested in poultry as defined in the specific Standard Requirement or Outline of Production for the product.

(3) The product is recommended for fish, other aquatic species, or reptiles. In such instances, the product shall be safety tested in fish, other aquatic species, or reptiles as required by specific Standard Requirement or Outline of Production for the product.

(c) Identity test. Methods of identification of Master Seed Bacteria to the genus and species level by laboratory tests shall be sufficient to distinguish the bacteria from other similar bacteria according to criteria described in the most recent edition of "Bergey's Manual of Systematic Bacteriology" or the American Society for Microbiology "Manual of Clinical Microbiology". If Master Seed Bacteria are referred to by serotype, serovar, subtype, pilus type, strain or other taxonomic subdivision below the species level, adequate testing must be used to identify the bacteria

to that level. Tests which may be used to identify Master Seed Bacteria include, but are not limited to:

(1) Cultural characteristics,

(2) Staining reaction,

(3) Biochemical reactivity,

(4) Fluorescent antibody tests,

(5) Serologic tests,

(6) Toxin typing,

(7) Somatic or flagellar antigen characterization, and

(8) Restriction endonuclease analysis.

(d) Ingredient requirements. Ingredients used for the growth and preparation of Master Seed Bacteria and of final product shall meet the requirements provided in 113.50. Ingredients of animal origin shall meet the applicable requirements provided in 113.53.

(e) Only serials tested for viricidal activity in accordance with the test provided in 113.35 and found satisfactory by such test shall be packaged as diluent for desiccated fractions in combination packages.

(f) If formaldehyde is used as the inactivating agent and the serial has not been found satisfactory by the viricidal activity test, bulk or final container samples of completed product from each serial shall be tested for residual free formaldehyde content using the Basic Fuchsin Test.

(1) The residual free formaldehyde content of biological products containing Clostridial antigens shall not exceed the equivalent of 0.5 percent formaldehyde solution (1,850 parts per million formaldehyde.)

(2) The residual free formaldehyde content of bacterins, bacterin-toxoids, and toxoids other than those containing Clostridial antigens, shall not exceed the equivalent of 0.2 percent formaldehyde solution (740 parts per million formaldehyde.)

[39 FR 16862, May 10, 1974. Redesignated at 55 FR 35562, Aug. 31, 1990, as amended at 60 FR 14355, Mar. 17, 1995]

Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Manual Determination of Formaldehyde in Veterinary Biologics (Schiff Test)

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Contact Person: Jan DeJong, 515-239-8511
email: jdejong@aphis.usda.gov

Approvals:

_____ Date:_____
P. Frank Ross, Head
Toxicology and Chemistry Section

_____ Date:_____
Arthur J. Davis, Chief
Pathobiology Laboratory

_____ Date:_____
Richard E. Hill, Quality Assurance Manager

_/s/ R. L. Levings_____ Date:_5/21/97__
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for the Manual Determination of Formaldehyde in Veterinary Biologics (Schiff Test)

Table of Contents

Supplemental Assay Method for the Manual Determination of Formaldehyde in Veterinary Biologics (Schiff Test)

1. Introduction

This laboratory procedure may be used for the colorimetric determination of free formaldehyde in killed viral and bacterial products. The test is based on the classical Schiff's reaction for determining aldehydes.

2. Materials

2.1 Equipment/instrumentation--Brand names are provided for reference only, equivalent equipment or materials may be used.

2.1.1 Spectrophotometer or colorimeter with cuvettes (Bausch and Lomb Spectronic 70)

2.1.2 Centrifuge capable of 800-1000 x G.

2.1.3 Routine laboratory glassware and supplies, including pipets, screw cap test tubes, conical screw cap centrifuge tubes, test tube racks, class A volumetric flasks, laboratory toweling or wipes, laboratory timer, beakers, pipetor bulb (repipetors are optional), graph paper, and adjustable ship curve

2.2 Reagents/supplies--All chemicals reagent grade unless specified. Store at room temperature, unless otherwise specified. All chemicals and solutions good for 1 year, unless specified.

2.2.1 Dilute hydrochloric acid, 2.5% (**NVSL Media 30036, specify formula and volume, Appendix 8.1**)

2.2.2 Formaldehyde Solution, 37% (Formalin)--Check formaldehyde content of reagent by USP method (**Reference 7.2**). Prepare 1% standard formalin solution by diluting 1 ml of 37% formaldehyde to 100 ml with water (use 100 ml volumetric flask).

2.2.3 Modified Schiff Reagent, (**NVSL Media 30019, Appendix 8.2**) This reagent remains satisfactory for use for several weeks, as long as it retains a strong odor of sulfur dioxide.

2.2.4 Water--Use deionized or distilled water or water of equivalent purity.

2.2.5 Emulsified samples (oil and water emulsion) require that the emulsion be broken before the test is run. Chemicals needed for this step:

1). Sodium chloride, 10%--(**NVSL Media 30192, specify formula and volume, Appendix 8.3**)

2). Chloroform

3. Preparation for the test

3.1 Personnel training--No specific training is required. Individual should have working knowledge of laboratory equipment listed in **Section 2**.

3.2 Equipment--Turn on spectrophotometer to allow the instrument to "warm up" for at least 30 minutes.

3.3 Reagents/controls--Prepare a 1% formalin solution by diluting 1 ml of 37% formaldehyde with water to 100 ml in volumetric flask. Use this solution as a control, diluting it as the standard solution and testing it at 2 levels (run 0.2% and 0.4% or levels that bracket the samples).

3.4 Sample--Reference the current version of **TCSOP0001** for sample submission.

Critical control point: Dilute the formalin standards and all samples to a constant ratio so that standards and samples are treated the same. The colorimetric reaction is very dependent upon time and temperature. The reaction is carried out at room temperature, consequently, it may be necessary to adjust the dilution to fit the conditions in each laboratory.

3.4.1 Dilute aqueous samples 1+3 (1+2 to 1+5) with water. Make duplicate dilutions.

3.4.2 Emulsified samples require that the emulsion be broken by adding 2 ml of sample to 4 ml of 10% NaCl and 6 ml of chloroform. This mixture is shaken for 30 seconds and then centrifuged for 5 minutes at 800-1000 x G. The clear upper layer is used in step 4.1.3. A separate standard curve should be prepared for these products in which the standards are treated the same as the emulsified samples.

4. Performance of the test

4.1 Samples

4.1.1 For each sample dilution, pipet 10 ml of 2.5% HCl into a tube and mark it (sample No._____).

4.1.2 Also for each sample dilution, pipet 12 ml of 2.5% HCl into a tube and mark it (sample blank No._____).

4.1.3 Place 1 ml of diluted sample into each of the tubes (**4.1.1 and 4.1.2**) and mix. Remember to make dilutions.

4.2 Standards

4.2.1 Pipet 2.5% HCl into a tube and mark the tube (standard blank). Use this tube to set the instrument at zero O.D.

4.2.2 Prepare tubes containing the 2.5% HCl and the diluted standard as described in the following table. Mark the tubes indicating the appropriate concentration of the standard.

Formalin equivalent(%)	0.1	0.2	0.3	0.4	0.5	0.6	0.7
ml of 2.5% HCl	10.9	10.8	10.7	10.6	10.5	10.4	10.3
ml of standard	0.1	0.2	0.3	0.4	0.5	0.6	0.7

4.2.3 Mix by gently inverting the tube.

4.3 In sequence, add 2 ml of Schiff Reagent to each tube marked sample or standard at 1 minute intervals. Do not add Schiff Reagent to any "blank" tubes. All tubes, at this point, should contain 13 ml.

4.4 Mix the tubes well but keep them in the proper sequence (mix immediately after the reagent is added).

4.5 Allow the color to develop for exactly 30 minutes from the time the first Schiff Reagent was added. Read O.D. at 570 nm and record the O.D. for each tube in sequence at 1 minute intervals.

4.6 Read and record the O.D. of each sample blank.

5. Interpretation of the test results

5.1 Draw a curve by plotting the O.D. of the standards versus the percent formalin.

5.2 Calculations

5.2.1 Subtract the O.D. of the sample blank from the O.D. of the sample. Record this difference.

5.2.2 Determine the percent formalin in the sample by comparing the net O.D. of the sample to the standard curve.

5.3 Retest

5.3.1 If the O.D. of the sample reads outside the end points of the standard curve, redilute the sample and retest (multiply results from the curve by this dilution factor).

5.3.2 If the color developed is extremely dark, redilute and retest. (If the color is faint, test at a lower dilution or check reagent, reagent may need to be replaced).

5.3.3 If the controls vary more than 10% from the expected value, make new standard and control solutions, redilute the sample, and retest.

6. Report of test results

6.1 Test results are reported following the current version of **TCSOP0001**.

7. References

7.1 Reference is made to the long term use of this procedure by the Toxicology and Chemistry Section.

7.2 U.S. Pharmacopia/National Formulary, current issue

7.3 This protocol is a revision of SAM 510, February 1, 1971, to the format presented here. Version .02 was generated to represent changes resulting from the creation of the Center for Veterinary Biologics. No technical changes from the previous version are represented.

Supplemental Assay Method for the Manual Determination of Formaldehyde in Veterinary Biologics (Schiff Test)

Appendix 8.1 NVSL media 30036

Author's Comment: Media 30036 is generic and open-ended for HCl solutions. The requestor must request volume needed as well as the formulation. In the case of this protocol, specifics are:

Prepare 1 liter by slowly adding 25 ml concentrated HCl to approximately 500 ml water in a 1 liter volumetric flask. Fill to volume.

Appendix 8.2 NVSL media 30019

(Retyped here to fit the space)

SCHIFFS REAGENT, MODIFIED (STERILITY)

DISSOLVE 0.05g OF BASIC FUCHSIN IN 90ML OF DISTILLED WATER. THEN ADD 1g OF SODIUM SULFITE, MIX UNTIL COMPLETELY DISSOLVED AND THEN ADD 1ML OF CONCENTRATED HCL AND DILUTE TO ONE LITER WITH WATER. KEEP SOLUTION IN WELL-STOPPERED AMBER BOTTLE.

Appendix 8.3 NVSL media 30192

Author's Comment: Media 30192 is generic and open-ended for NaCl solutions. The requestor must request volume needed as well as the formulation. In the case of this protocol, specifics are:

Prepare 100 ml by adding 10 g NaCl to approximately 50 ml water in a 100 ml volumetric flask. Dissolve and fill to volume.

ween applications. [NOTE—Make the streak as narrow as sible along the starting line, and keep within the 5-cm border.] inse the tip of the pipet with a drop of methanol–ammonia TS ant (9:1), and then streak the rinse along the starting line betw e 5- and 2.5-cm points at the right edge. Repeat the sing with two additional drops, and then blow out the pipet. Apply 10 µL of the *Mixed Standard Preparation* at the mark 25 cm from the left edge.

Place 30 mL of methylene chloride (mobile solvent) in a tray a 23- × 23- × 7.5-cm chromatographic chamber arranged for ascending chromatography (see *Chromatography* ⟨621⟩), and allow the chamber to equilibrate for about 15 minutes. Remove the cover, place from 7 to 10 mL of water in a second tray, and without delay suspend the prepared chromatographic paper sheet so that it dips into the mobile solvent. Cover and seal the chamber, and allow the chromatogram to develop for 1 hour. Remove the paper from the chamber, and allow to air-dry for 5 minutes. Place the chromatogram on a dry sheet of filter paper, and view under short-wavelength ultraviolet light. [NOTE—Conduct the following identification and marking without delay to avoid excessive exposure of the sulfonamide spots to ultraviolet irradiation.] Identify and mark the respective spots by matching R_f values with those of the spots produced by the *Mixed Standard Preparation*. [NOTE—Sulfadiazine and sulfamerazine are chromatographed with increasing R_f, respectively.]

Cut the marked zones from the paper, cut each zone into five or six pieces, and place the pieces from each spot in separate, well-stoppered, 50-mL flasks. Add 20.0 mL of dilute hydrochloric acid (1 in 100) to each flask, and allow to stand for about 30 minutes, swirling each flask at least five times during this period. Filter the solutions through dry glass wool into separate dry tubes, discarding the first 5 mL of filtrate. Transfer 5.0 mL of the subsequent filtrate from each solution into separate 10-mL volumetric flasks. Transfer 3.0 mL of each required *Standard Preparation* into separate, 10-mL volumetric flasks. To the flask, and to a blank flask containing 5 mL of dilute hydrochloric acid (1 in 100), add 1.0 mL of sodium nitrite solution (1 in 1000) and 0.10 mL of hydrochloric acid, and allow to stand for 3 minutes with frequent swirling. To each flask add 1.0 mL of ammonium sulfamate solution (1 in 200), and allow to stand for 3 minutes, swirling frequently. Finally, to each flask add 1.0 mL of freshly prepared *N*-(1-naphthyl)ethylenediamine dihydrochloride solution (1 in 1000), mix, dilute with water to volume, and mix. Allow each solution to stand between 15 and 60 minutes, then concomitantly determine the absorbances of the solutions in 1-cm cells, recording the spectra from 440 to 700 nm, in a suitable spectrophotometer, using the blank to set the instrument. Draw a baseline, and determine the corrected absorbance for each solution at the wavelength of maximum absorbance at about 545 nm.

Calculate the concentration, in mg per mL, of each sulfonamide in the *Assay Preparation* by the formula:

$$0.12C(A_U/A_S),$$

in which C is the concentration, in µg per mL, of the pertinent USP Reference Standard in the *Standard Preparation*, A_U is the corrected absorbance of the *Assay Preparation*, and A_S is the corrected absorbance of the pertinent *Standard Preparation*. From the concentration of the *Assay Preparation* thus determined, and applying appropriate dilution factors, calculate the percentage of sulfonamide in the specimen taken.

⟨531⟩ THIAMINE ASSAY

USP Reference Standards ⟨11⟩—*USP Thiamine Hydrochloride RS*.

The following procedure is provided for the determination of thiamine as an ingredient of Pharmacopeial preparations containing other active constituents.

Special Solutions and Solvents—

POTASSIUM FERRICYANIDE SOLUTION—Dissolve 1.0 g of potassium ferricyanide in water to make 100 mL. Prepare fresh on the day of use.

OXIDIZING REAGENT—Mix 4.0 mL of *Potassium Ferricyanide Solution* with sufficient 3.5 *N* sodium hydroxide to make 1000 mL. Use this solution within 4 hours.

QUININE SULFATE STOCK SOLUTION—Dissolve 10 mg of quinine sulfate in 0.1 *N* sulfuric acid to make 1000 mL. Preserve this solution, protected from light, in a refrigerator.

QUININE SULFATE STANDARD SOLUTION—Dilute 0.1 *N* sulfuric acid with *Quinine Sulfate Stock Solution* (39:1). This solution fluoresces to approximately the same degree as the thiochrome obtained from 1 µg of thiamine hydrochloride and is used to correct the fluorometer at frequent intervals for variation in sensitivity from reading to reading within an assay. Prepare this solution fresh on the day of use.

Standard Thiamine Hydrochloride Stock Solution—Transfer about 25 mg of USP Thiamine Hydrochloride RS, accurately weighed, to a 1000-mL volumetric flask. Dissolve the weighed Standard in about 300 mL of dilute alcohol solution (1 in 5) adjusted with 3 *N* hydrochloric acid to a pH of 4.0, and add the acidified, dilute alcohol to volume. Store in a light-resistant container, in a refrigerator. Prepare this stock solution fresh each month.

Standard Preparation—Dilute a portion of *Standard Thiamine Hydrochloride Stock Solution* quantitatively and stepwise with 0.2 *N* hydrochloric acid to obtain the *Standard Preparation*, each mL of which represents 0.2 µg of USP Thiamine Hydrochloride RS.

Assay Preparation—Place in a suitable volumetric flask sufficient of the material to be assayed, accurately weighed or measured by volume as directed, such that when diluted to volume with 0.2 *N* hydrochloric acid, the resulting solution will contain about 100 µg of thiamine hydrochloride (or mononitrate) per mL. If the sample is difficultly soluble, the solution may be heated on a steam bath, and then cooled and diluted with the acid to volume. Dilute 5 mL of this solution, quantitatively and stepwise, using 0.2 *N* hydrochloric acid, to an estimated concentration of 0.2 µg of thiamine hydrochloride (or mononitrate) per mL.

Procedure—Into each of three or more tubes (or other suitable vessels), of about 40-mL capacity, pipet 5 mL of *Standard Preparation*. To each of two of these tubes add rapidly (within 1 to 2 seconds), with mixing, 3.0 mL of *Oxidizing Reagent*, and within 30 seconds add 20.0 mL of isobutyl alcohol, then mix vigorously for 90 seconds by shaking the capped tubes manually, or by bubbling a stream of air through the mixture. Prepare a blank in the remaining tube of the standard by substituting for the *Oxidizing Reagent* an equal volume of 3.5 *N* sodium hydroxide and proceeding in the same manner.

Into each of three or more similar tubes pipet 5 mL of the *Assay Preparation*. Treat these tubes in the same manner as directed for the tubes containing the *Standard Preparation*.

Into each of the six tubes pipet 2 mL of dehydrated alcohol, swirl for a few seconds, allow the phases to separate, and decant or draw off about 10 mL of the clear, supernatant isobutyl alcohol solution into standardized cells, then measure the fluorescence in a suitable fluorometer, having an input filter of narrow transmittance range with a maximum at about 365 nm and an output filter of narrow transmittance range with a maximum at about 435 nm.

Calculation—The number of µg of $C_{12}H_{17}ClN_4OS \cdot HCl$ in each 5 mL of the *Assay Preparation* is given by the formula:

$$(A - b)/(S - d),$$

in which A and S are the average fluorometer readings of the portions of the *Assay Preparation* and the *Standard Preparation* treated with *Oxidizing Reagent*, respectively, and b and d are the readings for the blanks of the *Assay Preparation* and the *Standard Preparation*, respectively.

Calculate the quantity, in mg, of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the assay material on the basis of the aliquots taken. Where indicated, the quantity, in mg, of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) may be calculated by multiplying the quantity of $C_{12}H_{17}ClN_4OS \cdot HCl$ found by 0.9706.

⟨541⟩ TITRIMETRY

Direct Titrations—Direct titration is the treatment of a soluble substance, contained in solution in a suitable vessel (the titrate), with an appropriate standardized solution (the titrant), the end-

point being determined instrumentally or visually with the aid of a suitable indicator.

The titrant is added from a suitable buret and is so chosen, with respect to its strength (normality), that the volume added is between 30% and 100% of the rated capacity of the buret. [NOTE—Where less than 10 mL of titrant is required, a suitable microburet is to be used.] The endpoint is approached directly but cautiously, and finally the titrant is added dropwise from the buret in order that the final drop added will not overrun the endpoint. The quantity of the substance being titrated may be calculated from the volume and the normality or molarity factor of the titrant and the equivalence factor for the substance given in the individual monograph.

Residual Titrations—Some Pharmacopeial assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a "back titration." The quantity of the substance being titrated may be calculated from the difference between the volume of the volumetric solution originally added and that consumed by the titrant in the back titration, due allowance being made for the respective normality or molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

Chelometric Titrations—Simple, direct titrations of some polyvalent cations are possible by the use of reagents with which the cations form complexes. The titration of the calcium ion by this means is particularly advantageous, in comparison to the oxalate precipitation method previously used for Pharmacopeial purposes. The success of complexometry depends in large measure upon the indicator chosen. Often the color change of an indicator can be improved by the addition of a screening agent.

Titrations in Nonaqueous Solvents—Acids and bases have long been defined as substances that furnish, when dissolved in water, hydrogen and hydroxyl ions, respectively. This definition, introed by Arrhenius, fails to recognize the fact that properties characteristic of acids or bases may be developed also in other solvents. A more generalized definition is that of Brönsted, who defined an acid as a substance that furnishes protons, and a base as a substance that combines with protons. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

The apparent strength of an acid or a base is determined by the extent of its reaction with a solvent. In water solution all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to oxonium ion and the acid anion (leveling effect). In a weakly protophilic solvent such as acetic acid the extent of formation of the acetate acidium ion shows that the order of decreasing strength for acids is perchloric, hydrobromic, sulfuric, hydrochloric, and nitric (differentiating effect).

Acetic acid reacts incompletely with water to form oxonium ion and is, therefore, a weak acid. In contrast, it dissolves in a base such as ethylenediamine, and reacts so completely with the solvent that it behaves as a strong acid. The same holds for perchloric acid.

This leveling effect is observed also for bases. In sulfuric acid almost all bases appear to be of the same strength. As the acid properties of the solvent decrease in the series sulfuric acid, acetic acid, phenol, water, pyridine, and butylamine, the bases become progressively weaker until all but the strongest have lost their basic properties. In order of decreasing strength, the strong bases are sodium 2-aminoethoxide, potassium methoxide, sodium methoxide, and lithium methoxide.

Many water-insoluble compounds acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by nonaqueous titration. Furthermore, depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant. Pure compounds can be titrated directly, but it is often necessary to isolate the active ingredient in pharmaceutical preparations from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercuric acetate, which removes halide ion as the unionized mercuric halide complex and introduces the acetate ion.

For the titration of a basic compound, a volumetric solution of perchloric acid in glacial acetic acid is preferred, although perchloric acid in dioxane is used in special cases. The calomel-glass electrode system is useful in this case. In acetic acid solvent this electrode system functions as predicted by theory.

For the titration of an acidic compound, two classes of titrant are available: the alkali metal alkoxides and the tetraalkylammonium hydroxides. A volumetric solution of sodium methoxide in a mixture of methanol and toluene is used frequently, although lithium methoxide in methanol-benzene solvent is used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide.

The alkali error limits the use of the glass electrode as an indicating electrode in conjunction with alkali metal alkoxide titrants, particularly in basic solvents. Thus, the antimony-indicating electrode, though somewhat erratic, is used in such titrations. The use of quaternary ammonium hydroxide compounds, e.g., tetra-*n*-butylammonium hydroxide and trimethylhexadecylammonium hydroxide (in benzene-methanol or isopropyl alcohol), has two advantages over the other titrants in that (a) the tetraalkylammonium salt of the titrated acid is soluble in the titration medium, and (b) the convenient and well-behaved calomel-glass electrode may be used to conduct potentiometric titrations.

Because of interference by carbon dioxide, solvents for acidic compounds need to be protected from excessive exposure to the atmosphere by a suitable cover or by an inert atmosphere during the titration. Absorption of carbon dioxide may be determined by performing a blank titration. The blank should not exceed 0.01 mL of 0.1 *N* sodium methoxide VS per mL of solvent.

The endpoint may be determined visually by color change, or potentiometrically, as indicated in the individual monograph. If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with 0.1 *N* lithium perchlorate in glacial acetic acid for titrations in acidic solvents or potassium chloride in methanol for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the aqueous potassium chloride solution and residual potassium chloride, if any, by rinsing with water, then eliminating residual water by rinsing with the required nonaqueous solvent, and finally filling the electrode with the designated nonaqueous mixture.

The more useful systems for titration in nonaqueous solvents are listed in Table 1.

Indicator and Potentiometric Endpoint Detection—The simplest and most convenient method by which the equivalence point, i.e., the point at which the stoichiometric analytical reaction is complete, may be determined is with the use of indicators. These chemical substances, usually colored, respond to changes in solution conditions before and after the equivalence point by exhibiting color changes that may be taken visually as the endpoint, a reliable estimate of the equivalence point.

A useful method of endpoint determination results from the use of electrochemical measurements. If an indicator electrode, sensitive to the concentration of the species undergoing titrimetric reaction, and a reference electrode, whose potential is insensitive to any dissolved species, are immersed in the titrate to form a galvanic cell, the potential difference between the electrodes may be sensed by a pH meter and used to follow the course of the reaction. Where such a series of measurements is plotted correctly (i.e., for an acid-base titration, pH versus mL of titrant added; for a precipitimetric, complexometric, or oxidation-reduction titration, mV versus mL of titrant added), a sigmoid curve results with a rapidly changing portion (the "break") in the vicinity of the equivalence point. The midpoint of this linear portion or the inflection point may be taken as the endpoint. The equivalence point may also be

Table 1. Systems for Nonaqueous Titrations.

Type of Solvent	*Acidic* (for titration of bases and their salts)	*Relatively Neutral* (for differential titration of bases)	*Basic* (for titration of acids)	*Relatively Neutral* (for differential titration of acids)
Solvent[1]	Glacial Acetic Acid Acetic Anhydride Formic Acid Propionic Acid Sulfuryl Chloride	Acetonitrile Alcohols Chloroform Benzene Toluene Chlorobenzene Ethyl Acetate Dioxane	Dimethylformamide *n*-Butylamine Pyridine Ethylenediamine Morpholine	Acetone Acetonitrile Methyl Ethyl Ketone Methyl Isobutyl Ketone *tert*-Butyl Alcohol
Indicator	Crystal Violet Quinaldine Red *p*-Naphtholbenzein Alphazurine 2-G Malachite Green	Methyl Red Methyl Orange *p*-Naphtholbenzein	Thymol Blue Thymolphthalein Azo Violet *o*-Nitroaniline *p*-Hydroxyazobenzene	Azo Violet Bromothymol Blue *p*-Hydroxyazobenzene Thymol Blue
Electrodes	Glass-calomel Glass-silver–silver chloride Mercury–mercuric acetate	Glass-calomel Calomel-silver–silver chloride	Antimony-calomel Antimony-glass Antimony-antimony[2] Platinum-calomel Glass-calomel	Antimony-calomel Glass-calomel Glass-platinum[2]

[1] Relatively neutral solvents of low dielectric constant such as benzene, toluene, chloroform, or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.
[2] In titrant.

without plotting a curve. However, it should be noted that in asymmetrical reactions, which are reactions in which the number of anions reacting is not the same as the number of cations reacting, the endpoint as defined by the inflection of the titration curve does not occur exactly at the stoichiometric equivalence point. Thus, potentiometric endpoint detection by this method is not suitable in the case of asymmetric reactions, examples of which are the precipitation reaction,

$$2Ag^{+} + CrO_4^{-2}$$

and the oxidation-reduction reaction,

$$5Fe^{+2} + MnO_4^{-}.$$

All acid-base reactions, however, are symmetrical. Thus, potentiometric endpoint detection may be employed in acid-base titrations and in other titrations involving symmetrical reversible reactions where an indicator is specified, unless otherwise directed in the individual monograph.

Two types of automatic electrometric titrators are available. The first is one that carries out titrant addition automatically and records the electrode potential differences during the course of titration as the expected sigmoid curve. In the second type, titrant addition is performed automatically until a preset potential or pH, representing the endpoint, is reached, at which point the titrant addition ceases.

Several acceptable electrode systems for potentiometric titrations are summarized in Table 2.

Blank Corrections—As previously noted, the endpoint determined in a titrimetric assay is an estimate of the reaction equivalence point. The validity of this estimate depends upon, among other factors, the nature of the titrate constituents and the concentration of the titrant. An appropriate *blank correction* is employed in titrimetric assays to enhance the reliability of the endpoint determination. Such a blank correction is usually obtained by means of a *residual blank titration*, wherein the required procedure is repeated in every detail except that the substance being assayed is omitted. In such instances, the actual volume of titrant equivalent to the substance being assayed is the difference between the volume consumed in the residual blank titration and that consumed in the titration with the substance

Table 2. Potentiometric Titration Electrode Systems.

Titration	Indicating Electrode	Equation[1]	Reference Electrode	Applicability[2]
Acid-base	Glass	$E = k + 0.0591\ \text{pH}$	Calomel or silver–silver chloride	Titration of acids and bases
Precipitimetric (silver)	Silver	$E = E^{\circ} + 0.0591 \log [Ag^{+}]$	Calomel (with potassium nitrate salt bridge)	Titration with or of silver involving halides or thiocyanate
Chelometric	Mercury-mercury(II)	$E = E^{\circ} + 0.0296(\log k' - \text{pM})$	Calomel	Titration of various metals (M), e.g., Mg^{+2}, Ca^{+2}, Al^{+3}, Bi^{+3}, with EDTA
Oxidation-reduction	Platinum	$E = E^{\circ} + \frac{0.0591}{n} \log \frac{[\text{ox}]}{[\text{red}]}$	Calomel or silver–silver chloride	Titrations with arsenite, bromine, cerate, dichromate, hexacyanoferrate(III), iodate, nitrite, permanganate, thiosulfate

[1] Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode constant; k' = constant derived from Hg-Hg(II)-EDTA equilibrium; M = any metal undergoing EDTA titration; [ox] and [red] from the equation, ox + $ne \rightleftarrows$ red.
[2] Listing is representative but not exhaustive.

present. The corrected volume so obtained is used in calculating the quantity of the substance being titrated, in the same manner as prescribed under *Residual Titrations*. Where potentiometric end-point detection is employed, the blank correction is usually negligible.

⟨551⟩ ALPHA TOCOPHEROL ASSAY

The following procedure is provided for the determination of tocopherol as an ingredient.

Hydrogenator—A suitable device for low-pressure hydrogenation may be assembled as follows. Arrange in a rack or in clamps two 50-mL conical centrifuge tubes, connected in series by means of glass and inert plastic tubing and suitable stoppers of glass, polymer, or cork (avoiding all use of rubber). Use one tube for the blank and the other for the assay specimen. Arrange a gas-dispersion tube so that the hydrogen issues as bubbles at the bottom of each tube. Pass the hydrogen first through the blank tube and then through the specimen tube.

Procedure—Pipet into a suitable vessel 25 mL of the final washed ether solution of the unsaponifiable fraction obtained as directed for *When Tocopherol Is Present* under *Procedure* in the *Vitamin A Assay* ⟨571⟩, and evaporate to about 5 mL. *Without applying heat*, remove the remaining ether in a stream of inert gas or by vacuum. Dissolve the residue in sufficient alcohol to give an expected concentration of about 0.15 mg of alpha tocopherol per mL. Pipet 15 mL into a 50-mL centrifuge tube, add about 200 mg of palladium catalyst, stir with a glass rod, and hydrogenate for 10 minutes in the *Hydrogenator*, using hydrogen that has been passed through alcohol in a blank tube. Add about 300 mg of chromatographic siliceous earth, stir with a glass rod, and immediately centrifuge until the solution is clear.

Test a 1-mL aliquot of the solution by removing the solvent by evaporation, dissolving the residue in 1 mL of chloroform, and adding 10 mL of antimony trichloride TS: no detectable blue color appears. [NOTE—If a blue color appears, repeat the hydrogenation for a longer time period, or with a new lot of catalyst.]

Pipet 2 mL of the supernatant solution into a glass-stoppered, opaque flask, add 1.0 mL of a 1 in 500 solution of ferric chloride in dehydrated alcohol,* and begin timing the reaction, preferably with a stop watch. Add immediately 1.0 mL of a 1 in 200 solution of 2,2′-bipyridine in dehydrated alcohol, mix with swirling, add 21.0 mL of dehydrated alcohol, close the tube, and shake vigorously to ensure complete mixing. When about 9½ minutes have elapsed from the beginning of the reaction, transfer part of the mixture to one of a pair of matched 1-cm spectrophotometer cells. After 10 minutes, accurately timed, following the addition of the ferric chloride–dehydrated alcohol solution, determine the absorbance at 520 nm with a suitable spectrophotometer, using dehydrated alcohol as the blank. Perform a blank determination with the same quantities of the same reagents and in the same manner, but using 2 mL of dehydrated alcohol in place of the 2 mL of the hydrogenated solution. Subtract the absorbance determined for the blank from that determined for the assay specimen, and designate the difference as A_D.

Calculate the alpha tocopherol content, in mg, in the assay specimen taken by the formula:

$$30.2\ A_D/(LC_D),$$

in which A_D is the corrected absorbance, L is the length, in cm, of the absorption cell, and C_D is the content of the assay specimen in the alcohol solution employed for the measurement of absorbance, expressed as g, capsules, or tablets per 100 mL.

* NOTE—The absorbance of the blank may be reduced, and the precision of the determination thereby improved, by purification of the dehydrated alcohol that is used throughout the assay. Purification may be accomplished by the addition of a few crystals (about 0.02%) of potassium permanganate and of a few pellets of potassium hydroxide to the dehydrated alcohol, and subsequent redistillation.

⟨561⟩ VEGETABLE DRUGS—SAMPLING AND METHODS OF ANALYSIS

Sampling

In order to reduce the effect of sampling bias in qualitative and quantitative results, it is necessary to ensure that the composition of the sample used be representative of the batch of drugs being examined. The following sampling procedures are the minimum considered applicable to vegetable drugs. Some articles, or some tests, may require more rigorous procedures involving more containers being sampled and/or more samples per container.

Gross Sample—Where external examination of containers, markings, and labels indicates that the batch can be considered to be homogeneous, take individual samples from the number of randomly selected containers indicated below. Where the batch cannot be considered to be homogeneous, divide it into sub-batches that are as homogeneous as possible, then sample each one as a homogeneous batch.

No. of Containers in Batch (N)	No. of Containers to be Sampled (n)
1 to 10	all
11 to 19	11
>19	$n = 10 + (N/10)$

(Round calculated "n" to next highest whole number.)

Samples shall be taken from the upper, middle, and lower sections of each container. If the crude material consists of component parts which are 1 cm or less in any dimension, and in the case of all powdered or ground materials, withdraw the sample by means of a sampling device that removes a core from the top to the bottom of the container, not less than two cores being taken in opposite directions. For materials with component parts over 1 cm in any dimension, withdraw samples by hand. In the case of large bales or packs, samples should be taken from a depth of 10 cm because the moisture content of the surface layer may be different from that of the inner layers.

Prepare the gross sample by combining and mixing the individual samples taken from each opened container, taking care not to increase the degree of fragmentation or significantly affect the moisture content.

Laboratory Sample—Prepare the laboratory sample by repeated quartering of the gross sample. (NOTE—Quartering consists of placing the sample, adequately mixed, as an even and square-shaped heap and dividing it diagonally into four equal parts. The two opposite parts are then taken and carefully mixed. The process is repeated as necessary until the required quantity is obtained.)

The laboratory sample should be of a size sufficient for performing all the necessary tests.

Test Sample—Unless otherwise directed in the individual monograph or test procedure below, prepare the test sample as follows:

Decrease the size of the laboratory sample by quartering, taking care that each withdrawn portion remains representative. In the case of unground or unpowdered drugs, grind the withdrawn sample so that it will pass through a No. 20 standard-mesh sieve, and mix the resulting powder well. If the material cannot be ground, reduce it to as fine a state as possible, mix by rolling it on paper or sampling cloth, spread it out in a thin layer and withdraw the portion for analysis.

Foreign Organic Matter

Test Sample—Unless otherwise specified in the individual monograph, weigh the following quantities of the laboratory sample, taking care that the withdrawn portion is representative (quartering if necessary):

Roots, rhizomes, bark, and herbs	500 g
Leaves, flowers, seeds, and fruit	250 g
Cut vegetable drugs (average weight of the pieces is less than 0.5 g)	50 g

1.9.2.4 - Formaldehyde

If formaldehyde is used as the inactivating agent then a test for free formaldehyde should be carried out. Not more than 0.05% of free formaldehyde shall be present in the vaccine unless this higher concentration has been shown to be safe.

1.9.2.5 - Inactivation testing

A test for complete inactivation shall be performed on the harvest immediately after the inactivation procedure and, if applicable, the neutralisation or removal of the inactivating agent. The test selected should be appropriate to the vaccine bacteria being used and should consist of at least two passages in production media or in media prescribed in the European Pharmacopoeia.

No evidence of any live micro-organism should be observed.

1.10 - SAMPLES

Samples of all seed materials, reagents, in-process material and finished product shall be supplied to competent authorities, on request.

2 - FINISHED PRODUCT - ASSAY RESULTS REQUIRED IN THE THE APPLICATION FOR MARKETING AUTHORISATION

For each application, the results of the following tests shall be presented.

2.1 - SAFETY

Safety testing shall be carried out as specified in Directive 81/852/EEC, as modified by Directive 92/.../EEC and the indications given below.

The dose to be used shall be that quantity of the product to be recommended for use and containing the maximum titre or potency for which the application is submitted.

2.4.11. PHOSPHATES

To 100 ml of the solution prepared and, if necessary, neutralised as prescribed add 4 ml of *sulphomolybdic reagent R3*. Shake and add 0.1 ml of *stannous chloride solution R1*. Prepare a standard in the same manner using 2 ml of *phosphate standard solution (5 ppm PO_4) R* and 98 ml of *water R*. After 10 min, compare the colours using 20 ml of each solution.

Any colour in the test solution is not more intense than that in the standard.

2.4.12. POTASSIUM

To 10 ml of the prescribed solution add 2 ml of a freshly prepared 10 g/l solution of *sodium tetraphenylborate R*. Prepare a standard in the same manner using a mixture of 5 ml of *potassium standard solution (20 ppm K) R* and 5 ml of *water R*.

After 5 min, any opalescence in the test solution is not more intense than that in the standard.

2.4.13. SULPHATES

All solutions used for this test should be prepared with distilled water R.

Add 1 ml of a 250 g/l solution of *barium chloride R* to 1.5 ml of *sulphate standard solution (10 ppm SO_4) R1*. Shake and allow to stand for 1 min. Add 15 ml of the solution to be examined and 0.5 ml of *acetic acid R*. Prepare a standard in the same manner using 15 ml of *sulphate standard solution (10 ppm SO_4) R* instead of the solution to be examined.

After 5 min, any opalescence in the test solution is not more intense than that in the standard.

2.4.14. SULPHATED ASH

Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator and weigh. Place the substance to be examined in the crucible and add 2 ml of *dilute sulphuric acid R*. Heat at first on a water-bath, then cautiously over a flame, then progressively to about 600 °C. Continue the incineration until all black particles have disappeared and allow the crucible to cool. Add a few drops of *dilute sulphuric acid R*, heat and incinerate as before and allow to cool. Add a few drops of *ammonium carbonate solution R*. Evaporate and incinerate carefully, allow to cool, weigh, and repeat the ignition for periods of 15 min to constant mass.

2.4.15. NICKEL IN POLYOLS

Determine the nickel by atomic absorption spectrometry (*Method II, 2.2.23*).

Test solution. Dissolve 20.0 g of the substance to be examined in a mixture of equal volumes of *dilute acetic acid R* and *water R* and dilute to 100.0 ml with the same mixture of solvents. Add 2.0 ml of a saturated solution of *ammonium pyrrolidinedithiocarbamate R* (about 10 g/l) and 10.0 ml of *methyl isobutyl ketone R* and then shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solutions. Prepare three reference solutions in the same manner as the test solution but adding 0.5 ml, 1.0 ml and 1.5 ml respectively of *nickel standard solution (10 ppm Ni) R* in addition to the 20.0 g of the substance to be examined.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for preparation of the test solution omitting the substance to be examined. Measure the absorbance at 232.0 nm using a nickel hollow-cathode lamp as source of radiation and an air-acetylene flame.

The substance to be examined contains not more than 1 ppm of nickel, unless otherwise prescribed.

2.4.16. TOTAL ASH

Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator and weigh. Unless otherwise prescribed, evenly distribute 1.00 g of the substance or the powdered vegetable drug to be examined in the crucible. Dry at 100 °C to 105 °C for 1 h and ignite to constant mass in a muffle furnace at 600 °C ± 25 °C, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, filter through an ashless filter paper and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant mass.

2.4.17. ALUMINIUM

Place the prescribed solution in a separating funnel and shake with two quantities, each of 20 ml, and then with one 10 ml quantity of a 5 g/l solution of *hydroxyquinoline R* in *chloroform R*. Dilute the combined chloroform solutions to 50.0 ml with *chloroform R* (test solution).

Prepare a standard in the same manner using the prescribed reference solution.

Prepare a blank in the same manner using the prescribed solution.

Measure the intensity of the fluorescence (*2.2.21*) of the test solution (I_1), of the standard (I_2) and of the blank (I_3) using an excitant beam at 392 nm and a secondary filter with a transmission band centred on 518 nm or a monochromator set to transmit at this wavelength.

The fluorescence ($I_1 - I_3$) of the test solution is not greater than that of the standard ($I_2 - I_3$).

2.4.18. FREE FORMALDEHYDE

Use method A, unless otherwise prescribed. Method B is suitable for vaccines where sodium metabisulphite has been used to neutralise excess formaldehyde.

METHOD A

For vaccines for human use, prepare a 1 in 10 dilution of the vaccine to be examined. For bacterial toxoids for veterinary use, prepare a 1 in 25 dilution of the vaccine to be examined.

To 1 ml of the dilution, add 4 ml of *water R* and 5 ml of *acetylacetone reagent R1*. Place the tube in a water-bath at 40 °C for 40 min. Examine the tubes down their vertical axes. The solution is not more intensely coloured than a standard prepared at the same time and in the same manner using 1 ml of a dilution of *formaldehyde solution R* containing 20 µg of formaldehyde (CH_2O) per millilitre, instead of the dilution of the vaccine to be examined.

METHOD B

To 0.5 ml of a 1 in 100 dilution of the vaccine to be examined add 5 ml of a 0.5 g/l solution of *methylbenzothiazolone hydrazone hydrochloride R* and 0.05 ml of *polysorbate 80 R*, close the tube, shake and allow to stand for 60 min. Add 1 ml of *ferric chloride-sulphamic acid reagent R* and allow to stand for 15 min. The absorbance (*2.2.25*) of the solution measured at 628 nm using a reagent blank as compensation liquid is not greater than that of a standard prepared at the same time and in the same manner using 0.5 ml of a dilution of *formaldehyde solution R* containing 5 µg of formaldehyde (CH_2O) per millilitre.

If the vaccine to be examined is an emulsion, separate the aqueous phase by the following method. Add to the vaccine an equal volume of *isopropyl myristate R* and mix. To 3 volumes of the mixture add 2 volumes of *1 M hydrochloric acid*, 3 volumes of *chloroform R* and 4 volumes of a 9 g/l solution of *sodium chloride R*. Mix thoroughly. Centrifuge at 15 000 *g* for 60 min. Remove the aqueous phase and measure its volume. Use the aqueous phase for the test for formaldehyde described above, adjusting the concentration of formaldehyde in the standard to allow for the dilution of the vaccine during the separation of the phases. If the procedure described fails to separate the aqueous phase, add 100 g/l of *polysorbate 20 R* to the sodium chloride solution and repeat the procedure but centrifuging at 22 500 *g*.

2.4.19. ALKALINE IMPURITIES IN FATTY OILS

In a test-tube mix 10 ml of recently distilled *acetone R* and 0.3 ml of *water R* and add 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*. Neutralise the solution if necessary with *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide*. Add 10 ml of the oil to be examined, shake and allow to stand. Not more than 0.1 ml of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

2.4.20. ANTIOXIDANTS IN FATTY OILS

Examine by thin-layer chromatography (*2.2.27*) using *silica gel G R* as the coating substance. Use plates dried at 130 °C for 2 h.

Test solution (a). Dilute 20 g of the substance to be examined, taken from the centre of the sample, or 20 g of oil with 50 ml of *light petroleum R* and shake vigorously with two quantities, each of 30 ml, of methanol (75 per cent *V/V*). After clear separation of the two layers drain the lower layer representing the methanolic phase. Evaporate the combined methanol fractions under reduced pressure at as low a temperature as possible in an atmosphere of nitrogen. Dissolve the residue in 5 ml of *ethanol-free chloroform R*. Store in a well-closed container.

Test solution (b). Evaporate the light petroleum layer (upper layer) obtained in the preparation of test solution (a) carefully to dryness. Add 0.5 g of *pyrogallol R* dissolved in 100 ml of *ethanol R* and boil under a reflux condenser for 30 min with 15 ml of a freshly prepared 330 g/l solution of *potassium hydroxide R*. Allow to cool, dilute with 250 ml of *water R* and extract the unsaponifiable matter with three quantities, each of 100 ml, of *light petroleum R*. Wash the combined light petroleum extracts with *water R* until free from alkali and evaporate to dryness. Dissolve the residue in 5 ml of *ethanol-free chloroform R*. Store in a well-closed container.

A. NON-POLYHYDROXY ANTIOXIDANTS

Place the plate in a chromatographic tank with *ethanol-free chloroform R* until the solvent front has risen about 12 cm from the lower edge of the plate. Dry the plate in air for 20 min and for 20 min in a desiccator under vacuum.

Apply test solution (a) to starting point no. 1 as shown in Figure 2.4.20.-1 as a spot not more than 5 mm in diameter. The quantity to be applied depends on the concentration of the test solution. Generally it is between 2 µl and 10 µl. Apply on starting points no. 2 and no. 3 as shown in Figure 2.4.20.-1, 2 µl of a colour solution containing 0.1 g/l respectively of *dimethyl yellow R*, of *Sudan red G R* and of *indophenol blue R* in *benzene R*. Mark the path length (10 cm) on the plate for both running directions. Develop in the first running direction with *ethanol-free chloroform R*. Allow the plate to dry in air for 10 min, then turn it through 90° and develop in the second running direction with *benzene R*. Allow the plate to dry in air for 5 min and spray with a 200 g/l solution of *phosphomolybdic acid R* in *ethanol R* until a permanent yellow colour is obtained. Within 2 min blue spots begin to appear. After a further 5 min to 10 min, treat the plate with ammonia vapour until the background is clear white. The substances appear as blue, slightly violet or greenish spots. Evaluate the chromatogram by reference to Figure 2.4.20.-1. If a blue spot remains at the starting point carry out the separation and identification of polyhydroxy antioxidants (Method B).

B. POLYHYDROXY ANTIOXIDANTS

Apply separately to the plate 1 µl, 2 µl, 4 µl and 6 µl of test solution (a) and 1 µl to 2 µl of the colour solution. Develop over a path of 13 cm using a mixture of 30 volumes of *glacial acetic acid R*, 60 volumes of *benzene R* and 60 volumes of *light petroleum R*. Dry the plate in air, spray with a 200 g/l solution of *phosphomolybdic acid R* in *ethanol R* and continue visualisation as described for the identification of non-polyhydroxy antioxidants. The polyhydroxy antioxidants are identified by means of the position of the spots of the colour solution using Figure 2.4.20.-2.

C. ANTIOXIDANTS NOT EXTRACTABLE WITH METHANOL

Examine by thin-layer chromatography on a second plate using test solution (b) according to the method given for non-polyhydroxy antioxidants. Spray the plate with a 10 g/l solution of *dichloroquinonechlorimide R* in *ethanol R*. The spots become clearly visible within 15 min. Evaluate the chromatogram by reference to Figure 2.4.20.-1. The dotted zones correspond to α-tocopherol and butylated hydroxytoluene. Spots due to β-and γ-tocopherol appear in the zone corresponding to 2-(1,1-dimethylethyl)-4-methoxyphenol.

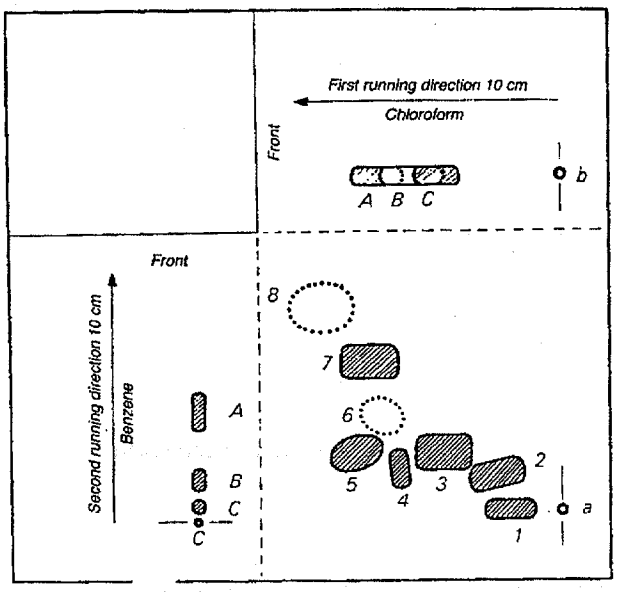

Figure 2.4.20.-1. — *Typical Chromatograms of Antioxidants (Methods A and C)*

a = Starting point no. 1 + gallates spot + nordihydroguaiaretic acid spot

b = Starting point no. 2

c = Starting point no. 3

1 = Guaiacum resin

2 = 3-(1,1-Dimethylethyl)-4-methoxyphenol

3 = 2-(1,1-Dimethylethyl)-4-methoxyphenol

4 = 2,2,5,7,8-Pentamethyl-6-chromanol

5 = Tetraethylthiuram disulphide

6 = α-Tocopherol

7 = Dibutylhydroxyanisole

8 = Butylhydroxytoluene

A = yellow

B = red

C = blue

Method A: continuous line

Method B: dotted line

If butylated hydroxytoluene is present in substantial quantities it may be detected by Method A.

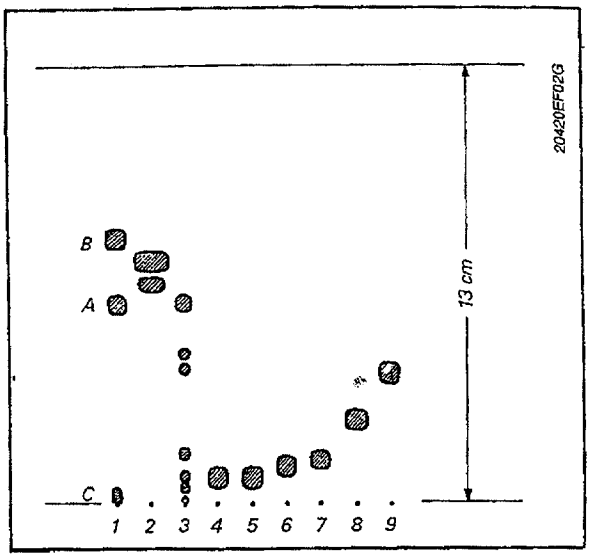

Figure 2.4.20.- 2. — *Typical Chromatograms of Polyhydroxy Antioxidants (Method B)*

1 = Colour solution

2 = Butylated hydroxyanisole

3 = Guaiacum resin

4 = Nordihydroguaiaretic acid

5 = Methyl gallate

6 = Ethyl gallate

7 = Propyl gallate

8 = Octyl gallate

9 = Dodecyl gallate

A = yellow

B = red

C = blue

2.4.21. FOREIGN OILS IN FATTY OILS BY THIN-LAYER CHROMATOGRAPHY

Examine by thin-layer chromatography (*2.2.27*) using *kieselguhr G R* as the coating substance. Impregnate a plate by placing it in a chromatographic tank containing the necessary quantity of a mixture of 10 volumes of *liquid paraffin R* and 90 volumes of *light petroleum R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen by at least 12 cm from the lower edge of the plate, remove the plate and allow the solvent to evaporate for 5 min. Carry out the chromatography in the same direction as the impregnation.

Preparation of the mixture of fatty acids. Heat 2 g of the oil with 30 ml of *0.5 M alcoholic potassium hydroxide* under a reflux condenser for 45 min. Add 50 ml of *water R*, allow to cool, transfer to a separating funnel and extract with three quantities, each of 50 ml, of *ether R*. Discard the ether extracts, acidify the aqueous layer with *hydrochloric acid R* and extract with three quantities, each of 50 ml, of *ether R*. Combine the ether extracts and wash with three quantities,

This is the Japanese Standard

Assay method of formalin

This is a method to determine the content of formalin in the sample from the absorbance at a wavelength of 410 nm utilizing that formaldehyde in formalin reacts acetylacetone and ammonia under slight acidic conditions, produces 3,5-diacetyl 1,4-dihydrolutidine and develops an orange-yellow color.

1. Standard solution and test solution

1.1. Standard formalin solution

Dilute formaldehyde solution exactly 500-fold with water.

1.2. Acetic acid-ammonium acetate buffer solution (pH 6.25)

1.2.1. Acetic acid solution

To 12.9 mL of acetic acid, add water to make 100 mL.

1.2.2. Ammonium acetate solution

Dissolve 173.4g of ammonium acetate in water to make 1,000 mL.

Mix 40 mL of the acetic acid solution and 1,000 mL of the ammonium acetate solution and store in a cold and dark place.

1.3. Acetylacetone test solution

Mix 7 mL of acetylacetone and 14 mL of dehydrated ethanol and add water to make 1,000 mL.

2. Test method

Dilute the test sample exactly with water so as to contain formalin in 0.01 to 0.05% and use this solution as the sample. Pipet 0.5, 1, 1.5, 2 and 2.5 mL of the standard formalin solution and add water to make exactly 10 mL. Use these solutions as the standard dilutions of 0.01, 0.02, 0.03, 0.04 and 0.05 vol%.

Pipet 0.1 mL each of the sample and each standard dilution, add 2 mL each of the acetic acid-ammonium acetate buffer solution, mix with 2 mL each of the acetylacetone test solution, and warm at 60℃ for 15 minutes. Cool these solutions with cold water for 5 minutes and allow to stand for 20 minutes. However, when the solution is turbid, centrifuge this solution at 1400×g or higher for 10 minutes. Determine the absorbance at a wavelength of 410 nm.

Prepare the calibration curve from the absorbance of the standard dilutions and extrapolate the absorbance of the sample to determine the content of formalin in the test sample. Separately, perform a blank determination using water as the control in a similar manner to determine the absorbance and make any necessary correction.